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5 City of Chesterfield, State of Missouri, whose post office address is 437 Hunters Hill Drive,
Chesterfield, Missouri 63017, have invented new and useful improvements in

PHARMACOLOGICAL AGENT AND METHOD OF TREATMENT

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation-in-part of application Serial No. 09/127,620, filed August 1, 1998, which is a continuation-in-part of application Serial No. 08/843,157, filed April 11, 1997, now abandoned, which is a continuation in part of application Serial No. 08/581,351, filed December 29, 1995, now U.S. Patent No. 5,767,135, and which claims priority to provisional application Serial No. 60/024,221, filed October 22, 1996 and to provisional application Serial No. 60/026,992, filed September 20, 1996.

STATEMENT REGARDING FEDERALLY

SPONSORED RESEARCH OR DEVELOPMENT

None

BACKGROUND OF THE INVENTION

The invention relates to the treatment of and immunization against proliferative diseases, inflammatory diseases and pain. More specifically the invention relates to the use of metal chelating materials including, picolinic acid, fusaric acid, their derivatives, analogs and related chemicals as pharmacological and/or biological response modifier agents.

It will be appreciated that hereinafter the use of the term "response modifier" is intended to encompass all of the intended functions of the invention and method including antiviral, antiinfective, antiinflammatory, anticancer, vaccine and so on. Further, it will be appreciated that the broad term "antiinfective" is intended to include antibacterial, antifungal, antiparasitic functions, as well as actions against any other infective agent or organism including viruses not encompassed by the term "antiviral". It will also be appreciated that the term "antiinflammatory" is intended to include an inflammatory response modifier, including

all inflammatory responses such as production of stress proteins, white blood cell infiltration, fever, pain, swelling and so forth. Furthermore, the term "analgesic" is intended to include a pain reliever, whether the pain incurred is a result of disease, inflammation, trauma or psychosomatic reaction.

5 Researchers recently have come to appreciate the role of metal containing proteins in physiological actions and responses including cancer, pain, inflammation, proliferative and infectious diseases. Generally speaking, the inventor has studied the important function of proteins having amino acid sequences which bind metals, particularly transition metal ions therein. For example, the inventor has determined the important role zinc finger or zinc ring proteins as hormone-receptor proteins and in proliferative, inflammatory and infectious diseases. Moreover, the inventor has determined the role of other metal ion containing protein complexes, such as the role of iron finger proteins such as iron-finger hormone-receptor proteins in aging and carcinogenesis.

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20 The inventor and others have recognized at least three efficient approaches to inhibiting zinc finger proteins: 1) disruption of the zinc finger by modification of the cysteines which are at least one of the four binding sites for Zn^{2+} in the zinc finger protein which results in the ejection of zinc ion; 2) removal of the zinc from the zinc finger moiety by specific chelating agents; and 3) specific chelating agents that form a ternary complex at the site of zinc binding on zinc finger proteins, resulting in inhibition of the DNA or RNA binding activity of zinc finger proteins.

Papilloma virus infection results in a number of proliferative diseases in subjects including warts induced by type 4 human papilloma virus (common warts). Moreover, papilloma virus can cause plantar ulcers as well as plantar warts. Human papilloma virus

infection of the uterine cervix is the most common of all sexually transmitted diseases. Commonly know as genital warts, this wide spread virus infection is a serious disease that potentially can develop into cervical cancer. Since the virus is permanently present in cells, infection recurs in a significant percentage of patients. In many instances, conization of the
5 uterine cervix is required to remove the infected tissue.

Condylomata acuminata, also denoted genital warts, are benign epithelial growths that occur in the genital and perianal areas and caused by a number of human papilloma viruses (HPV) including types 6,11 and 54. These are low risk viruses which rarely progress to malignancy. However, high risk viruses such as HPV-16 and HPV-18 are associated with
10 cervical intraepithelial cancer.

The actions of HPV are mediated by specific viral-encoded proteins which interact and/or modulate cellular DNA and proteins to produce abnormal growth and differentiation of cells. Two proteins of the HPV viral genome, E6 and E7, are well conserved among anogenital HPV's and both may contribute to the uncontrolled proliferation of basal cells
15 characteristics of the lesions. The E7 oncoprotein is a multi-functional protein with transcriptional modulatory and cellular transforming properties. The E7 oncoprotein is denoted as a "zinc finger" protein because it possesses a sequence motif that is implicated in zinc binding. A strong correlation between zinc binding and the transactivation activity of E7 has been documented. The HPV-16 E6 protein is a "zinc finger" protein that binds DNA and
20 may have transcriptional properties such that its function may be dependent upon the formation of zinc fingers. E6 protein can complex with the cellular tumor suppressor protein p53 and it is necessary with E7 protein for the immortalization of primary human squamous cells. Only two proteins of HPV are consistently expressed and integrated in keratinocytes,

the E6 and E7 zinc finger proteins. The E6 and E7 proteins are responsible for continuous cell proliferation. About twenty HPVs are associated with ano-genital lesions and all transformed keratinocytes of these lesions contain E6 and E7 zinc finger proteins. The E6 and E7 regulate growth and transformation by interfering with cellular p53 and pRb proteins, respectively. Thus, one should be able to control or cure HPV by inactivating E6 and E7, the critical zinc finger proteins which are required for replication. When replication of the virus is halted, apoptosis of the virally-infected cells must occur. Thus, one can alter the epidemiology of, for example, carcinoma of the uterine cervix by interfering with the function of zinc finger or zinc ring proteins.

Herpes viruses, for example, Herpes Simplex Virus (HSV), has two important viral metalloproteins, a zinc finger protein and ribonucleotide reductase, an iron-containing enzyme, which are necessary for replication and propagation of the viruses. One can alter the course of herpes diseases, such as “fever blisters” and genital herpes, by inhibiting the two viral metalloproteins.

The human immunodeficiency virus (HIV) encodes several regulatory proteins that are not found in other retroviruses. The tat protein, which is one of these proteins, trans-activates genes that are expressed from the HIV long terminal repeat and tat is essential for viral replication. The tat protein of the HIV-1 is a zinc finger protein that when added to certain cells in tissue culture, specifically promotes growth. It has been shown that the tat protein of HIV-1 stimulates growth of cells derived from Kaposi's sarcoma lesions of AIDS patients. Other experiments raised the possibility that tat might act as a viral growth factor to stimulate replication in latently infected cells or alter expression of cellular genes.

The nucleocapsid p7 protein of HIV has been targeted by the inventor for treatment of HIV viral infections. The p7 protein is required for the correct assembly of newly formed virus particles during the viral life cycle. Moreover, the p7 protein contains two zinc fingers that are required for the recognition and packaging of the viral RNA. Because the zinc finger domain is essential for nucleic acid binding, p7 resistant mutants are highly unlikely to occur. Thus, agents that effectively attack the two zinc finger domains of the HIV virus nucleocapsid p7 in vivo will decrease the overall number of viral particles that bud off and exit the cells to infect other cells.

The hepatitis C virus is not integrated with DNA and thus may be vulnerable to attack by specific antivirals. The hepatitis C viruses are dependent upon the Zn 2+ metalloproteinases for specific viral functions. Processing at the C terminus portion of the NS2 protein of hepatitis C virus is mediated by virus encoded protease (metalloproteinases). Modification of the metalloproteinases presents an opportunity for controlling the progression of hepatitis C mediated disease.

It is of interest to note that the breast cancer susceptibility gene BRCA 1 includes a zinc ring domain that are involved in protein-protein interactions or protein-DNA interactions. It also is of interest to note that the zinc ring domain of the BRCA 1 has a 54% sequence similarity and 38% sequence identity with a zinc ring domain encoded by the genome of the equine herpes virus. (R. Bienstock, "Molecular Modeling of Proteins Structures, Science & Medicine, January/February 1997, p.56).

From the foregoing it appears that it would be beneficial to have a product that can interfere with the formation or action of certain zinc finger proteins or zinc ring proteins to stop the progress of certain virally induced or mediated proliferative diseases or to halt the

progress of viruses or malignancies dependent upon zinc finger or zinc ring proteins for their transformation and immortalization. Furthermore, it would be beneficial to provide a product that can halt the growth of other proliferative cells, such as malignant cells by chelating metal ions from zinc-dependent or iron-dependant, transition metal ion (e.g. copper, iron, etc.) dependent proteins, hormones and enzymes necessary for the replication of the malignant cells.

Furthermore, since the products can chelate metals, such as iron, they have a role in metal toxicity states, such as iron or lead toxicity.

BRIEF SUMMARY OF THE INVENTION

It is among the objects of the present invention to provide a compound which can retard the growth and proliferation of target viruses or cells by blocking the activity of metal ion-containing proteins and proteases.

Another object of the present invention to provide a compound which can retard the growth and proliferation of target infective organisms including bacteria, fungi, parasites or other infective agents by blocking the activity of metal ion-containing proteins.

Another object of the present invention is to provide a compound which can retard the growth and proliferation of target viruses or cells by blocking the activity of transition metal ion-containing protein structures such as zinc finger, zinc ring proteins, iron-finger or iron-ring proteins or enzymes associated with replication.

Yet another object of the invention is to provide such a compound that can be added to foods, pharmaceuticals or other perishables as a preservative.

Another object of the invention is to provide such a compound to retard angiogenesis, particularly angiogenesis in tumors and in the eye following ophthalmologic surgery.

Yet another object of the invention is to provide such a compound that inhibits fibroblast production after cataract surgery which results from an inflammatory response to artificial lenses.

It is another object of the present invention to provide a compound that can retard the growth of premalignant and malignant cells such as virally, chemically and spontaneously transformed cells.

Another objects of the present invention to provide a compound that can retard the growth of premalignant and malignant cells such as virally, chemically and spontaneously transformed cells and be administered by any acceptable route, including orally, with substantial effectiveness and minimal side effects.

It is also among the objects of the present invention to provide a new treatment for patients suffering from various forms of spontaneous and retroviral-induced proliferative diseases and cancers by utilizing the novel properties of metal chelating agents as a chemotherapeutic/anti-viral agent and/or biological response modifier.

It is another object of the present invention to provide an agent that can halt the proliferation and transmission of the HIV virus.

It is another object of the invention to provide a method of halting the function of zinc finger proteins, zinc ring proteins, and other proteins with zinc binding motifs heretofore unidentified by the administration of a zinc chelating agent, both topically and systemically.

Another object of the invention to provide a method of halting the function of metal containing protein structures containing metals other than zinc, metal-containing ring proteins structures, such as iron-finger or iron-ring proteins and other proteins with metal binding

motifs heretofore unidentified by the administration of a metal chelating agent, both topically and systemically

Another object of the invention is to provide a product which can be spray in the nostrils or inhaled to prevent or control upper respiratory diseases such as influenza, colds or pulmonary cancer.

Another object of the invention is to provide an antiinflammatory compound that is effective in a broad range of inflammatory disorders including symptoms of well-know autoimmune diseases as well as inflammatory response to infections and to chemical assault or radiation including, but not limited to, ultraviolet, atomic or medical radiation.

Yet another object of the invention is to provide such chelating agents in a relatively safe and nontoxic form such as picolinic acid. its derivatives or related or similar compounds for both topical and systemic use.

Another object of the invention is to provide a topical preparation of metal chelating agents such as picolinic acid or its derivatives to treat virally induced or spontaneous proliferative diseases of the skin or mucous membranes in human and animal subjects.

It is still another object of the present invention to provide an intravaginal preparation containing metal chelating agents such as picolinic acid or derivatives thereof that can prevent or retard sexually transmitted diseases caused by viruses or other causative agents containing zinc finger proteins or zinc ring proteins.

Still another object of the present invention is to provide a preparation containing chelating agents such as picolinic acid or derivatives thereof that halts the progression of viral infections or proliferative diseases that is non-toxic to normal cells, easy to use, relatively inexpensive and well suited for its intended purposes.

According to the invention, briefly stated, a method or treatment and compound used in the method, for example, metal chelating compounds, such as picolinic acid or derivative thereof, for the treatment of infective or proliferative diseases, actinic lesions, inflammatory response, radiation assault, and cancers in human and animal subjects, as well as the method of treatment. The invention can be used orally or topically to treat or control a wide assortment of proliferative diseases or conditions, both spontaneous or induced by viruses, bacteria, fungi, chemicals, ultraviolet light for example. The metal chelating compounds bind metal, for example iron or transition metal ions such as zinc, required by enzymes, heat shock proteins or by transcription proteins found in viruses or malignant cells. By way of further example, the metal chelating compound, for example picolinic acid or its derivatives, is used to bind the zinc-containing p7 protein common to the HIV virus, thereby inactivating the virus and preventing the exit of RNA containing viruses or particles from the cells.

Moreover, the invention can be added to foods, fresh fruits, pharmaceutical agents and other perishables as preservative. The invention will exert its antifungal and antibacterial effects on the perishables, but is applied in a low, non-toxic concentration.

Another embodiment of the invention includes approximately 3 to 6 mM picolinic acid or derivative in an isotonic solution for intranasal use or inhalation to treat or prevent upper respiratory diseases.

Also, picolinic acid in 500 mg capsules given in dosages ranging from 250 mg per day to 2000 mg per day, or more, has been shown to be effective in reducing the size of tumors, such as cancerous lymph nodes and inducing necrotic tissue in the tumor.

One embodiment of a topical preparation consists of a solution of the chelator, for example, 0.01% to 99%, preferably 5% to 25%, picolinic acid in an appropriate vehicle, such

as deionized water, lotion or so forth, and is applied to the lesion one or two times a day. The preparation can be applied to skin to control acne, warts and herpes infections and to toe nails and finger nails, for example, to treat fungal infections. In another embodiment, the topical preparation consists of an ointment or cream containing approximately 0.5% to 99%,
5 preferably 5% to 20% picolinic acid which is applied once or twice daily to the lesion and to a bandage placed on the lesion. The ointment or cream can be instilled intravaginally to retard sexually transmitted viral diseases.

The various embodiments of the topical preparation can be used to treat papilloma and herpes viral diseases and to retard the papilloma, herpes and HIV 1 viruses as well as
10 proliferative diseases such as psoriasis, actinic lesions and skin cancer.

Various active derivatives that maintain their activity and stability when after systemic administration are provided. Slow release oral formulations can be used to treat diseases for the digestive tract. The active derivatives can be administered orally, parenterally, by inhalation, transdermally or by any other appropriate method to control proliferative diseases,
15 cancers, viral infections, HIV, and any other condition wherein the causative agent includes a zinc-containing protein, whether the zinc-containing protein is a zinc finger protein, a zinc ring protein, or other type of zinc or metal containing structure heretofore unidentified or undetected, wherein the metal containing segment is required for protein stability and configuration.

20 It will be appreciated that other appropriate chelating materials such as the derivative of picolinic acid or fusaric acid or even unrelated compounds, also may be used. It also will be appreciated that, although 5% to 20% topical preparations of the picolinic acid are described, a broader range of concentrations may be used. For example from approximately

0.001% to 99.9% metal chelating agent may be used. Further, the systemic doses may be altered or adjusted to ranges greater or lesser than those described, depending on toxicity and patient response, without departing from the scope of the appended claims.

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWINGS

5 In the drawings, Figure 1 is the chemical structure of fusaric acid ;
Figure 2 illustrates the effect of picolinic acid on total protein of LoVo cells;
Figure 3A illustrates the effects of fusaric acid on the growth of WI-38 cells;
Figure 3B illustrates the effects of fusaric acid on the growth LoVo cells;
Figure 3C illustrates the effects of fusaric acid on the growth of KB cells;
10 Figure 4A illustrates the effects of fusaric acid on morphology of WI-38 cells, the cells treated without fusaric acid;
Figure 4B illustrates the effects of fusaric acid on morphology of WI-38 cells, the cells treated with fusaric acid;
Figure 4C illustrates the effects of fusaric acid on morphology of LoVo cells, the cells
15 treated without fusaric acid;
Figure 4D illustrates the effects of fusaric acid on morphology of LoVo cells, the cells treated with fusaric acid;
Figure 5A illustrates the effects of fusaric acid on morphology of KB cells, the cells treated without fusaric acid;
20 Figure 5B illustrates the effects of fusaric acid on morphology of KB cells, the cells treated with fusaric acid;
Figure 6 illustrates modulation of apoptosis by intracellular concentrations of zinc;
Figure 7 illustrates the role of zinc finger proteins in HIV infection;

Figure 8 illustrates the effect of picolinic acid and derivatives on HIV, zinc finger proteins and retroviruses;

Figure 9 illustrates disruption of zinc finger binding domains in retroviral proteins caused by picolinic acid;

5 Figure 10 illustrates the wide spectrum of antiviral activity of picolinic acid;

Figure 11 illustrates the molecular structure of a derivatives of picolinic acid for system use;

Figure 12 illustrates the binding of a picolinic acid derivative to zinc and adjacent amino acids of a zinc finger protein; and

10 Figure 13 is a schematic illustrating the interrelation of viral infection, ribosomal proteins and the zinc finger heat shock protein, DnaJ which is involved in inflammation.

DETAILED DESCRIPTION OF THE INVENTION

15 Picolinic acid, a metal chelating, naturally occurring, biological compound, which inhibits the growth of numerous cultured normal and transformed mammalian cells. Picolinic acid has the chemical name of 2-Pyridine carboxylic acid, also known as alpha-pyridine carboxylic acid, having the chemical formula $C_6H_5HO_2$, molecular weight: 123.11 g/mol, and is readily soluble in water. Picolinic acid has an LD-50 of 140 grams in a 70 Kg subject.

20 It has been shown that short-term treatment with picolinic acid arrests normal cells in G_1 , (G_0) while transformed cells are blocked in different phases of the cell cycle. With longer exposure to picolinic acid cytotoxicity and cell death was observed in all transformed cells whether they were blocked in G_1 , G_2 or at random. In contrast, most normal cells showed no toxic effects from the picolinic acid. Thus, the selective growth arrest and the differential

cytotoxicity induced by picolinic acid reveals a basic difference in growth control and survival mechanism(s) between normal and transformed cells.

Kinetic and radiosotopic studies show that picolinic acid both inhibits incorporation of iron into the cells and effectively removes radioiron from the cells. Hence, it is conceivable that the inhibition of cell proliferation *in vitro*, as well as tumor growth *in vivo*, by picolinic acid results, at least in part, from selective depletion of iron in the cells.

However, it also is shown that picolinic acid may arrest prokaryote and eukaryote cell growth by inhibiting Zn-requiring enzymes. In addition to its chelating ability, picolinic acid has a number of biologic properties such inhibitory effects on ADP ribosylation and ribosomal RNA maturation, modulation of hormonal responses, and macrophage activation. Picolinic acid in combination with interferon gamma can inhibit retroviral J2 mRNA expression and growth in murine macrophages. Thus, picolinic acid and its derivatives can act as a biological response modifier.

The inventor has determined that picolinic acid and fusaric acid were found to inhibit the zinc dependent binding of recombinant MPS-1 to DNA, as determined by gel shift assays and the data correlates with the absence of radioactive Zn65 from recombinant MPS-1 protein. MPS-1 is a ubiquitous tumor marker and cell growth stimulator and is described in detail in the inventor's United States Patent No. Re.35,585 (No. 5,243,041). MPS-1 has one zinc finger domain of the type CCCC. Picolinic acid and fusaric acid react with the CCCC zinc finger to remove radioactive Zn65 from MPS-1. This is detected by a change in the electrophoretic mobility of MPS-1 under non-denaturing conditions. These experiments indicate that picolinic acid and derivatives should remove zinc and denature various types of zinc finger or zinc ring proteins, whether known or heretofore undiscovered, including viral

proteins such as nucleocapsid p7 proteins, as will be explained further. Furthermore, the inventor has determined that any chemical compound, whether known or heretofore undiscovered, that will remove the zinc (or other metal) and denature the proteins or that will form a ternary complex (protein-zinc-chelator) can be effective as a therapeutic agent or as an autologous immune response modulator, as will be discussed in greater detail below.

Fusaric acid is a potent inhibitor of cancerous cell growth. Fusaric acid, a picolinic acid derivative, metal ion chelator, shows an effect on the growth and viability of normal and cancerous cells in tissue culture. Examples presented here show that fusaric acid has potent anti-cancer and anti-viral activity *in vitro*. Moreover, fusaric acid may be useful in the treatment of spontaneous and virally-induced tumors *in vivo* without substantially damaging living normal cells.

Fusaric acid is the 5-butyl derivative of picolinic acid. Its structure is shown in Fig. 1. Fusaric acid was recognized in the early 1960's to have activity as an antihypertensive agent *in vivo*. Fusaric acid and its properties can be summarized as follows: Undoubtedly the drug interacts with various metalloproteins and metal ion-requiring enzyme systems. Fusaric acid is noted to be an inhibitor of a wide variety of seemingly unrelated enzyme systems. These include poly ADP ribose polymerase, a Zn-finger enzyme, and other Zn-finger proteins. Cu-requiring systems are also effected by fusaric acid. These enzymatic systems are important in growth control mechanisms. It has become increasingly clear that fusaric acid, by virtue of its butyl group penetrates the cell interior much more easily than picolinic acid, and works at least in part as a Zn/Cu chelating agent.

As mentioned above, the hepatitis C family of viruses are dependent upon metalloproteinases having a zinc finger domain for replication of the virus. Picolinic acid,

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fusaric acid or other suitable derivatives or analogs, can be administered orally to patients exposed to or suffering from hepatitis C –related disease to bind the metal in the metalloproteinases and thereby control the disease. Furthermore, the oral administration of the metal chelator in combination with interferons will result in the elimination of the virus from the cells because the hepatitis C virus is not integrated with the DNA and thus is vulnerable to this double attack. A pharmaceutically active and acceptable preparation of picolinic acid or derivative in a concentration of approximately 1% to approximately 99%, preferably in a daily range of approximately 250mg to 6000 mg ,preferably approximately 500 mg to approximately 2000 mg of picolinic can be used for this mode of treatment. It will be appreciated that doses approximating the LD-50 of 140 grams/70 Kg may be covered by the invention in the event continued research shows higher doses are optimal.

Novel substituted derivatives of picolinic acid and related compounds can be used systemically to treat cancer, viral infections and other related diseases and proliferative disorders. The novel substituted derivatives of picolinic acid and related compounds also work by disrupting the binding of zinc atoms in zinc finger proteins, zinc ring proteins or other structures heretofore unknown that depend upon the inclusion of zinc or other metal ions such as transition metal ions, for stability, packaging or replication. Further, the novel substituted derivatives are stable and retain their zinc chelating properties even when introduced systemically by injection, oral administration, inhalation or transdermal or other routes of administration

Fig 11 illustrates novel derivatives of picolinic acid for systemic use. Computer modeling indicates that such derivatives can interact with zinc atoms and disrupt its binding to the zinc finger protein. Substitutions at positions 3, 4,5 and 6 on the 2-pyridine carboxylic

acid (picolinic acid) have the proper configuration to prevent interference with the zinc finger protein backbone. For example R1, R2, R3 or R4 can be a methyl, ethyl, propyl, isopropyl, butyl, isobutyl, secondary butyl, tertiary butyl, pentyl, isopentyl, neopentyl or similar group. Further, substitution with halogens such as fluorine, chlorine, bromine and iodine can result in effective, systemically active agents. The systemic compounds can be prepared by methods generally known to the art and include pharmacologically acceptable salts thereof.

Figure 12 illustrates the binding of zinc in a zinc finger or zinc ring protein by derivatives of picolinic acid. Further, as shown in Figure 12, the substituted positions at positions 3, 5 or 6 i.e. R1, R3 or R4 can attach to amino acids on each side of the zinc thus binding the zinc containing protein at three sites and forming ternary complex comprised of the protein, the zinc, and the picolinic acid derivative which inactivates the protein. Therefore, the above-listed moieties that can be substituted at the various positions can result in a picolinic acid derivative that not only is more stable for systemic administration, but also one that has even greater affinity and specificity for, and binding potential with, various zinc finger or zinc ring proteins.

It will be appreciated that substitutions at the 3,4,5 and 6 positions can be made with a peptide of sixteen amino acids or more with either basic or acid amino acids predominating. The substituted picolinic acid would have an increased molecular weight and a substantially increased half-life in the blood. Further, such compounds would penetrate the virus-containing cells more effectively due to the amphipathic nature of the peptide residues.

The systemic compounds can be administered to human and animal subjects by any means that produces contact of the active agent with the target protein, such as orally, parenterally, inhalation, transdermally, rectally, on any other method for obtaining a

pharmacologically acceptable blood level. In general, a pharmacologically effective daily
does can be from about 0.01 mg/kg to about 25 mg/kg per day or any other pharmacologically
acceptable dosing. A pharmaceutically active and acceptable preparation of picolinic acid or
derivative in a concentration of approximately 1% to approximately 99%, preferably in a
5 daily range of approximately 250mg to 6000 mg ,preferably approximately 500 mg to
approximately 2000 mg of picolinic can be used for this mode of treatment. It will be
appreciated that doses approximating the LD-50 of 140 grams/70 Kg may be covered by the
invention in the event continued research shows higher doses are optimal.

It will be appreciated that *in vivo* administration of picolinic acid or its derivatives for
10 the treatment of cancer, for example, has unexpected results, not predicted by the effect of
fusaric acid or picolinic acid on cells *in vitro*, as described below in the examples. The
inventor has determined that *in vivo* , the compounds enhance production of natural killer T-
cells which enhances the effect on cancer cells and the like, that is not observed *in vitro*.

The products, such as picolinic acid, fusaric acid and derivatives, may be used to
15 removed metals from a subject in disease states such as Wilson's disease, iron overload or
lead poisoning. Oral doses or injectable doses in the broad range of 250 mg to less than 140
grams per day. Optimally, a dosage of 500 mg to 2000 mg per day would be used, with
dosages up to 6000 mg or more in resistance cases.

It will be appreciated that picolinic acid derivatives referred to herein as the systemic
20 compounds can be employed in the hereinafter described topical preparations as well as
employed systemically. Furthermore, the claimed invention is intended to include any other
chemical compounds, either derivatives of picolinic acid, compounds with structural
relationships to picolinic acid, or heretofore unknown compounds that function to chelate,

attach to, or modify metal ions in proteins structures, including, but not limited to transition metal ions found in proteins structures of viruses, proliferative cells (plant or animal) or even as components of fungi and bacteria.

It previously has been discovered that p7 protein is required for correct assembly of newly formed virus particles during the viral life cycle, as explained above. By modeling, the inventor has discovered the activity of picolinic acid in disrupting zinc finger proteins in retroviruses, as is illustrated in FIGS. 6-10. FIG. 6 illustrates modulation of apoptosis by intracellular concentrations of zinc; FIG. 7 illustrates the role of zinc finger proteins in HIV infection; FIG. 8 illustrates the effect of picolinic acid and derivatives on HIV, zinc finger proteins and retroviruses; FIG. 9 illustrates disruption of zinc finger binding domains in retroviral proteins caused by picolinic acid; and FIG. 10 illustrates the wide spectrum of antiviral activity of picolinic acid.

The p7 protein contains two zinc fingers that are required for the recognition and packaging of viral RNA. In one embodiment and aspect of the invention, the inventor has targeted p7 for drug therapy with picolinic acid and derivatives. Picolinic acid and derivatives are zinc finger disrupting agents that act by attacking the two zinc finger domains of the virus (i.e. HIV) nucleocapsid p7 in vitro. This results in picolinic acid and derivatives inducing an overall decrease in the number of viral particles that bud off and exit the cells to infect other cells. It is known that HIV-1 contains two zinc fingers in the retroviral p7 protein. The zinc fingers are highly conserved throughout essentially all retroviruses. Thus, mutations in the zinc fingers of the HIV-1 virus will produce a non-infectious HIV-1 particle. Because the zinc finger domain is essential for nucleic acid binding, p7 resistant mutants will not occur. The picolinic acid can be used, therefore, for prevention of retroviral and other viral diseases

by, for example, inhibiting exit of the virus or virus particles from the cells or by chemically inducing a non-infectious virus. Furthermore, any chemical entity, either known or unknown at this time, that functions in the same manner as picolinic acid or its derivatives, is intended to be encompassed by the instant invention. Representative viruses which include zinc finger or zinc ring proteins are included on Table 1.

TABLE 1
Examples Of Families Of Viruses Using Zinc Finger Proteins, Zinc Ring Proteins Or
Transition Metal Ion-Dependent Enzymes For Replication And/Or Virulence

Virus protein and Mr	Location and general Characteristics	Protein Function and Specific Properties
Reovirus		
Lambda-1, 140 Kd	Inner capsid	Zinc finger protein
		Binds dsDNA
Rho-3, 41Kd	Outer capsid	Zinc finger protein
		Binds dsRNA
Rotavirus		
NSP1, 53 Kd	Non-structural	Zinc finger protein
		RNA binding
Retroviridae		
Ncp7 (AIDS)	Nucleocapsid	Zinc finger protein
55 amino acids		RNA binding
		Required for inclusion of RNA in virions
TAT (AIDS)	Regulatory protein	Cluster of 7 cystein residues
82-101 amino acids		Trans-activator
Papillomavirus		
E6	Regulatory protein	Zinc finger protein
		Transforming protein of HPVs
		Continuous cell proliferation
		Targets degradation of p53
E7	Regulatory protein	Zinc finger protein
		Transforming protein of HPVs
		Continuous cell proliferation
		Binds to the retinoblastoma protein, Rb
Adenovirus		
E1A	Regulatory protein	Zinc finger protein
		Gene expression
		Transforming protein
Hepatitis C		

NS2(+NS3)	Zn-dependent enzyme	Zn-metalloproteinase
Herpes viruses		
HSV-1: ICPO protein	Regulatory protein	Zinc finger
		DNA-binding
		Trans-activation
HSV-2: MDBP protein	Regulatory protein	Zinc finger protein
		ssDNA-binding
		DNA replication
ICP6: Ribonucleotide	Fe-dependent	Synthesis of DNA precursors
Reductase	Enzyme	
Equine Herpes virus-1	Regulatory protein	Zinc ring configuration
ZR protein		DNA binding
		Protein/protein interactions

PRESERVATIVES

The chelating agents of the present invention can be used as preservatives in perishable items such as foods and pharmaceuticals and to prevent fungal growth on the surface of fresh fruits. Presently chemicals such as citrashine orthophenilphenol thiabendazole are used. Stability experiments have shown that picolinic acid, for example, is highly stable when used as a preservative in foods and on the surface of fresh fruits. Microbial and fungal growth is inhibited while the food components are unaffected. The preservative of the present invention has less likelihood of toxicity or untoward reactions if ingested than the present, complex chemical antifungals. The preferred concentration of picolinic acid or acceptable derivative for this use is approximately below 0.025% to be classified as a preservative. Of course, greater percentages, e.g. up to 99.9% also would be effective.

ANGIOGENESIS

The chelating agents of the present invention are used to control angiogenesis. New blood vessels are formed because copper Cu^{2+} is available to stimulate certain enzymes. Angiogenesis can be problematic in two specific situations. First, the increased blood vessel formation in tumors and the increased blood vessel formation in the eye, particularly after

ophthalmologic surgery. Increased angiogenesis, in combination with increased fibroblast production, as will be discussed in greater detail below, can result in opacity of the ocular lens. The administration of the novel chelating agent, particularly picolinic acid, fusaric acid or acceptable derivatives, analogs or related products, prevent unwanted angiogenesis. In the case of tumor control, the chelating agent can be administered orally or injected directly into the tumor. In the treatment of the eye, the product can be administered orally or preferably, topically to the eye. An ophthalmic preparation for control of angiogenesis includes approximately 0.01% to approximately 99% active ingredient, most preferably 5% to 10%. Systemic administration in approximately 250 mg to less than 140 g per day, preferably 250 mg to 2000 mg is used to control angiogenesis.

HEAT SHOCK PROTEINS, VIRAL INFECTION

AND INFLAMMATORY RESPONSE

Prokaryotes and eukaryotes express numerous heat shock proteins (Hsps) in response to stress, including heat shock, exposure to heavy metals, hormones and viral infections. These heat shock proteins mediate and exacerbate the inflammatory response. Furthermore, Hsp27, the most common hsp found in mammals and has been shown to be involved in cancer, such as breast cancer. An increase in cellular levels of Hsp27 and Hsp70 increase the resistance of cancer cells to apoptosis.

The stress response which includes numerous forms of physiological and pathological stresses is involved in viral infection also. A prominent feature of this response is the synthesis of a discrete set of proteins, known as the heat shock proteins which, at present, are denoted molecular chaperons. The role of these proteins are illustrated schematically in Figure 13. During infection by certain viruses, heat shock proteins act as intracellular detectors that

recognize malformed proteins. In general aberrant protein folding and degradation reactions caused by exogenous or endogenous factors have emerged as a cause of cancer and aging. Researchers have found that viruses are able to activate heat shock proteins. For example, Hsp70 (DnaK) is induced by adenovirus, herpes virus, cytomegalovirus, and other viruses.

5 DnaJ proteins are zinc finger proteins, defined by the J domain, which is essential for stimulation of the Hsp70 (DnaK) ATPase activity. Thus, the results suggest that there may be a relationship between the stress response and the cytopathic effects of certain viruses such as herpes viruses. Hsp70 has a protective role in inflammation, infection and a regulatory role in cytokine biosynthesis. Hsp70 exists in cells in equilibrium between its free state, in the
10 cytoplasm, and its bound state, protecting proteins in the nucleolus, interacting with ribosomal proteins to either refold some of the unfolded ribosomal proteins or by solubilizing the denatured ribosomal proteins to facilitate their use and increase turnover rate. During release from heat shock and as the nucleolus begins to recover its normal activities, a significant portion of Hsp70 returns to the cytoplasm. The inventor believes this protein-protein
15 interaction has profound implications for viral replication, since viruses control ribosomal protein synthesis and during such process many of them damage the cells. Hence cellular inflammatory responses to viral infection are part of the organism defense against viruses. The compounds and methods of the present invention can be used to block the action of the DnaJ zinc finger proteins when excessively expressed. By blocking the DnaJ zinc finger
20 proteins, which are essential for stimulation of the Hsp70 (DnaK) ATPase activity, the Hsp70 destroying activity of Hsp70 ATPase enzyme will be inhibited, thus blocking the effect of the Hsp70. This blocking of the DnaJ zinc finger protein which is required for the enzyme activity, therefore, will reduce the stress reaction in virally infected cells.

Furthermore, it is believed that the response of cells to stress, such as exposure to UV radiation, chemicals, bacteria, parasites or fungi is associated with the induction of heat shock proteins. The inventor has successfully used formulations of 1%, 5% or 10% picolinic acid in an appropriate vehicle, such as an absorption base, to treat sunburn in over 25 cases. Hence, the compounds and methods of the present invention can be used to block inappropriate and excessive cellular stress response caused, in this example, by UV radiation. Moreover, the inventor has successfully treated the inflammation associated with acne in more than 35 cases, in both adults and teenagers, with a 5% to 10% aqueous solution of picolinic acid. As determined by computer modeling, the compound binds with DnaJ and reduces or eliminates the effect of the DnaJ blocking zinc finger proteins inactivating enzymes and hence controls the inflammatory response induced by bacteria in common acne.

Presently recognized disease states involving inflammatory responses that may be susceptible to the inventor's methods also include arthritis, Alzheimer's disease. Furthermore, Arteriosclerosis has an inflammatory and proliferative component which may be blocked by the instant compositions and methods. By way of further example, it is known that inflammatory cytokines play some role in Alzheimer's disease. Inflammatory molecules and mechanisms are present in the disease and inflammation may be an essential component of the disease sufficient to cause neurodegeneration. Thus, suitable doses of the disclosed picolinic acid derivatives, particularly those shown to cross the blood-brain barrier, can be used to treat the inflammatory component of the disease.

Furthermore, the claimed invention is intended to apply to other pathological conditions which involved inflammatory responses both presently known and unknown. Another example of a salutary use of the claimed invention is to prevent fibroblast production

in the eye after cataract surgery and the implantation of artificial lenses, particularly those made from polymeric materials. The lenses, due to surface contamination, induce proliferation of fibroblasts with resultant opacity of the lens. Presently, 5 fluorouracil is instilled in the eye to control fibroblast proliferation. However, the 5fluorouracil is toxic and not without untoward effects. Topical application of the chelating agents of the present invention, specifically as discussed below, can control fibroblast proliferation. The combination of the antifibroblast activity and the antiangiogenesis activity make chelating agents, such as fusaric acid, ideal drugs after ophthalmologic surgery.

It will be appreciated by those skilled in the art that the inventor has disclosed the best mode by which he presently understands picolinic acid and its derivatives, to function in controlling inflammatory response. However, the scope of the appended claims is intended to include other mechanisms of action, both presently known and unknown, which include metal ion containing proteins as mediators in the inflammatory response including, but not limited to, parasitic diseases such as toxoplasmosis and malaria.

VIRUS AND CANCER VACCINE

It is clearly established that zinc finger proteins of viruses are essential for viral replication and that mutations of the zinc finger domain produce non-infective viral particles. Thus, the zinc finger domain is an essential component of the virus, without which it cannot replicate, exit from the cells and infect other cells. Moreover, since the zinc finger domain is highly conserved, it generally does not mutate. However, when and if the zinc finger domain does mutate, the mutation is lethal, that is, the virus dies.

Based upon the foregoing understanding of the function and activity of metal ion containing proteins (metalloproteins) , the inventor has discovered a method of preparing

vaccines using metalloproteins essential for virus replication and packaging of viruses. In general, a virus is treated with the metal chelating agent, picolinic acid or one of the afore described derivatives or other suitable derivatives, for example, which denatures the zinc finger proteins of the virus. The picolinic acid, for example, distorts the protein configuration to such an extent that it becomes immunogenic and new antigenic sites are exposed. Thus, the patient develops antibodies against viral replicases and viral proteins involved in packaging the viruses.

Generally speaking, the method includes an antigenic approach to virus and cancer treatment using zinc finger protein-antigen complexes derived from an individual patient's tumors or virus-containing tissues as well as zinc finger protein-antigen complexes derived from non-autologous tissue sources. The resulting vaccines are used to stimulate a cellular immune response against viruses, primary tumor cells as well as metastasis.

More specifically, in method of the present invention the DNA or RNA is removed by standard nucleic acid technology and discarded. After removal of the nucleic acid, the viral metalloproteins are mixed with the picolinic acid or a derivative thereof in an emulsion to prevent renaturation of proteins by binding to metal ions. The zinc finger proteins are captured by beads derivatized with an analog of picolinic acid containing a side chain at the 5 position, or other suitable position, of sufficient length to allow binding of the protein to the picolinic acid metal ion. The zinc finger proteins then are conjugated to an immunogenic protein such as keyhole limpet hemocyanin (KLH), for example as taught in U.S. Patent 5,243,041 (Re. 35,583). The final antigen, consisting of the KLH-zinc finger protein-picolinic acid derivative. The picolinic acid derivative is covalently bonded to the zinc finger protein

to prevent renaturization of the zinc finger protein. The proteins are injected into animals following standard schedules.

Vaccines against certain viruses, particularly against HIV are difficult to produce. The ineffective nature of the hosts natural immune response indicates that a vaccine effective against HIV must provide highly specific protective immunity and, more important, must provide sterilizing immunity. This problem is compounded by the large number of HIV-1 strains. In fact, HIV-1 can be considered a *quasi-virus* that mutates continuously and thus vaccines for one strain are not cross-reactive or only minimally neutralizing for different strains, even in the same patient. However, the present method of preparing a vaccine using a highly conserved zinc finger protein derived from HIV will have cross antigenicity and will overcome problems associated with known methods of vaccinating against HIV.

It will be appreciated also that metalloproteins have been shown to play an important role in cancer. Metalloproteins, such as MPS-1 have growth stimulating functions that may be implicated in cancer cell proliferation and in metastasis. Thus, a vaccine, prepared as described above with reference to viral metalloproteins can be used to immunize patients against the deleterious effects of zinc finger proteins or other metalloproteins that play a role in cancer cell growth and proliferation.

It will be appreciated that various changes and modifications may be made in the preparations and methods described and illustrated without departing from the scope of the appended claims. It will be appreciated that the description of the specific embodiments of the preparation for topical use is intended to include pharmacologically accepted concentrations of the above described substituted derivatives of picolinic acid, as well as the picolinic acid itself. Oral or injectable forms of the preparations also are contemplated by the

invention. Further, suitable preparations, other than topical preparations, of metal chelating compounds may be employed for the treatment of adenocarcinomas and squamous cell carcinomas. The preparation may be used alone or in combination with other chemotherapeutic agents. The picolinic acid or derivative can be included with various chemical or mechanical carriers, both known and heretofore unknown, to allow penetration or entry into tumors. Furthermore, the preparations may be used to treat a wide spectrum of proliferative and viral diseases mediated by zinc finger proteins, zinc ring proteins or other metal ion dependent proteins or enzymes. Therefore, the foregoing specification and accompanying drawings are intended to be illustrative only and should not be view in a limiting sense.

ZINC-FINGER AND IRON-FINGER HORMONE RECEPTOR PROTEINS AND AGING AND CARCINOGENESIS

At physiological concentrations, transition metal ions, such as iron, cobalt and copper are essential elements for biological functions; at higher levels, however, they are toxic. This is particularly true for iron. Toxicity of the transition metal ions, particularly iron, is the fact that protein domains are present within key enzyme and transcriptional regulatory molecules (DNA-binding proteins) which normally bind zinc (zinc finger domains) but which can substitute zinc by other transition by other transition metals that are present in the cell.

Elevated levels of iron contribute to carcinogenesis in several ways; iron has the capacity to generate highly reactive free radicals that damage DNA, and rapidly proliferating transformed cells have increased requirement for iron for DNA replication (ribonucleotide reductase) and for energy production by mitochondria.

Iron can replace zinc in the zinc-containing hormone-receptor proteins for testosterone, progesterone and other hormones. Iron may also generate free radicals which damage DNA in specific regulatory regions and potentially induce carcinogenesis in the prostate, uterus, and other organs. Thus, classical hormones can modulate iron-finger receptor proteins. The hormones potentiate the destructive actions of free radicals, mediated by abnormal iron-finger receptor proteins, on regulatory regions of DNA. The inventor determined that it is feasible to maintain zinc-finger proteins in an undamaged zinc-containing form by using a combination of specific agents, such as iron chelators and radical scavengers, respectively, interfere with the formation of both aberrant iron-finger proteins and free radicals. Thus, picolinic acid, fusaric acid, and pharmacologically acceptable derivatives thereof, in the dosages discussed above, as well as chelators yet unknown, can be used to prevent the formation of aberrant iron-finger proteins involved in carcinogenesis and aging. Free radical scavengers include known anti-oxidants such as vitamin E and so forth.

Examples of the specific effects of metal chelating agents, including picolinic acid, substituted picolinic acid derivatives and fusaric acid, as well as the practical application of those agents will now be described:

EXAMPLE 1

Effects of Picolinic Acid on Growth of WI-38, LoVo and KB, Cells

Cells were plated at 1.5×10^5 cells/60-mm dish; 48 hours later, the medium was removed, and new media with or without 3 mM picolinic acid were added. Total cell protein was determined at the indicated times; each point is the average of triplicate measurements from 2 cultures.

The growth of normal WI-38 cells was inhibited by 3 mM picolinic acid within 24 hours, the cells showed no toxic effects for up to 72 hours of treatment, and the inhibition was reversible within 24 hours of removal of the agent (data not shown). These results are identical to previous results with WI-38 cells incubated with picolinic acid.

5 The growth of LoVo cells was inhibited by 3 mM picolinic acid (Fig. 2). After 24 to 48 hours of exposure to picolinic acid (3 mM), LoVo cells acquired a flattened morphology, they began to look granular, no mitosis were observed, and some began to float in the medium, (data not shown). With longer exposure (48-72 hours) cytotoxicity and cell death was observed in LoVo cells (data not shown). Equivalent results were obtained with
10 cancerous KB cells treated with picolinic acid (3 mM) but its cytotoxic effects on this cell type were not as pronounced as in the case of LoVo cells (data not shown).

EXAMPLE 2

Effect of Fusaric Acid on Growth and Viability of Normal WI-38 cells.

In initial experiments to examine the effects of fusaric acid on cell growth and
15 viability, WI-38 and LoVo cells were incubated for 24 to 72 hours in medium with or without various doses of fusaric acid (0.1-1 mM). The growth of both WI-38 and LoVo cells was inhibited by 500 μ M fusaric acid in a time and dose dependent manner, as shown below in Table 2. A higher dose of fusaric acid (1 mM), caused a pronounced decrease in the rate of cell growth of both cell lines, and extensive cytotoxicity was noted particularly in LoVo cells
20 by 24 hours. These preliminary experiments led to detailed tests of the effects of the highest dose of fusaric acid (500 μ M) which appeared to show some differential toxicity on LoVo cells with little toxicity to WI-38 cells (Table 2).

Table 2
Effect of Different Doses of Fusaric Acid on
WI-38 and LoVo Total Cell Protein

	Addition	Monolayer Protein ($\mu\text{g}/\text{dish}$) ^a			
		0h	24h	48h	72h
10	WI-38				
	None	105	202	270	371
	Fusaric acid (0.5 mM) --		195	275	345
	Fusaric acid (1 mM) --		236	202	195
15	LoVo				
	None	202	270	352	457
	Fusaric acid (0.5mM) --		135	90	101
	Fusaric acid (1mM) --		ND	ND	ND

^aCells were plated at 1.5×10^5 cells/60-mm dish in DME/F12 medium containing 10% Calf serum. The medium was removed 24 hours later and then fresh media containing the indicated concentrations of fusaric acid were added. Protein was determine at the indicated times. Points are the mean of duplicate determinations. SE did not exceed 5% of the mean. ND, not done because of extensive cell destruction.

Fig. 3A shows that the growth of WI-38 cells was strongly inhibited by 500 μM fusaric acid. After 30 to 48 hours in 500 μM fusaric acid, WI-38 cells acquired a more flattened morphology, showed some granularity, and no mitotic cells , as illustrated in Fig. 3B, or further increase in cell number were observed (See, Fig. 3A). Following 30 hours incubation with fusaric acid (500 μM), normal growth rate was not restored after removal of fusaric acid and the cell number decreased significantly (30%) after 4 days in normal media. The remaining cells were spread on the substratum in normal manner without any visible mitosis for 4 days after removal of the drug. However, they resumed growth after 125 hours

of removal of fusaric acid (Fig. 3A), and most (>95%) of the cells survived. These results suggest that the majority of WI-38 cells were arrested in G₁(G₀) by fusaric acid and they proceeded slowly through the cell cycle after its removal.

To examine WI-38 cell viability in greater detail, the effects of fusaric acid were studied in logarithmically growing and contact inhibited confluent cells (Tables 2 and 3). In logarithmically growing WI-38 cells approximately 76% of the cells were viable after 30 hours of treatment with fusaric acid. When the cells were treated for 78 hours, only 26% of the cell population survived the pronounced cytotoxic actions of fusaric acid. The data are shown below in Table 3.

Table 3
Viability of Cells in Logarithmic Growth After Treatment with Fusaric Acid^a

Cell line	% Survival ^b	
	30h	78h
WI-38		
Control	100	100
Treated	76.4	26
LoVo		
Control	100	100
Treated	38.5	4.5

^aThe cells were incubated in medium with or without 500 μ M fusaric acid for the indicated times.

^bFraction of total cells counted which did not stain with trypan blue. Cells attached to the dish were exposed to trypan blue and counted. The percentage exclusion by untreated cultures was normalized to 100% for comparison with fusaric acid-treated cultures.

The detach cells showed conspicuous cytotoxic effects and most of them were destroyed. Interestingly, in confluent cell, fusaric acid did not show any cytotoxic effects as determined by the fact that 100 % of the cells survived 48 hours of treatment with 500 μ M fusaric acid , as shown in Table 4, below.

Table 4

Viability of Confluent Cells after Treatment with Fusaric Acid (500 μ M)

Cell line	% Survival ^a	
	Control	Treated
WI-38	100	100
LoVo	100	40
KB	100	95

^aDetermined at 48h using trypan blue dye exclusion test as indicated in Table 3.

Thus, a significant proportion of the population of growing cells (76%) and all of the confluent WI-38 cells cell resisted the marked cytotoxic action of fusaric acid.

EXAMPLE 3

Effect of Fusaric Acid on Growth and Viability of Colon Carcinoma LoVo Cells.

Fusaric acid (500 μ M) inhibited LoVo cell growth, as shown in Fig. 3B. After 30 hours of treatment with 500 μ M fusaric acid, there was a prominent decrease in cell number. DNA synthesis was completely (100%) inhibited by 24 hours. When treated with 500 μ M fusaric acid, the majority of the LoVo cells acquired a rounded morphology by 48 hours.

As shown in Fig. 4D, most of the cells became granular, showed pronounced cytotoxic effects, many were destroyed, and subsequently detached from the culture dish. These floating cells were not viable. They did not adhere to the substratum and disintegrated after 1 to 3 days when resuspended in fresh medium without fusaric acid. Fig. 4B shows that within

30 hours of treatment there was a 60% decrease in cell number. Following removal of the drug after 30 hours of treatment showed that the cell population continued to decline (~ 80%) in number up to approximately 100 hours (Fig. 4B). However, after 100 hours, an increase in cell number was noted after 25 additional hours.

5 As in the case of WI-38, LoVo cell viability after fusaric acid treatment was investigated in logarithmically growing and confluent cells, as shown in Tables 2 and 3. In logarithmically growing LoVo cells, approximately 38% of the attached cells were viable after 30 hours of treatment with fusaric acid. When the cells were treated for 78 hours, only 4.5% of the cell population survived the pronounced cytotoxic actions of fusaric acid. The detach cells showed noticeable cytotoxic and most of them were destroyed at these time points. In confluent cell, fusaric acid showed a significant cytotoxic effect as determined by the fact that only 40% of the cells survived 48 hours of treatment with 500 μ M fusaric acid. Thus, LoVo cells are much more sensitive to the cytotoxic actions of fusaric acid in both growing and confluent population of cells in comparison to normal WI-38 cells.

EXAMPLE 4

Effect of Fusaric Acid on Growth and Viability of Human Carcinoma KB Cells.

Fig 2C shows that the growth of KB cells was inhibited by fusaric acid (500 μ M). After 24 hours of treatment there was no further increase in cell number. As illustrated in Figs. 3C and 5B, after 24-48 hours in 500 μ M fusaric acid, most of the KB cells acquired a more flattened morphology, and no mitotic cells or further increase in cell number were observed. Following 48 hours incubation with fusaric acid (500 μ M), normal growth rate was not restored after removal of fusaric acid and the cell number decreased significantly after 27 additional hours in normal media (Fig. 3C). The remaining cells were spread on the

substratum in normal manner without any visible mitosis for 27 additional hours after drug removal. However, they resumed growth after 27 hours of removal of fusaric acid (Fig. 3C).

To examine KB cell viability in greater detail, the effects of fusaric acid were studied in logarithmically growing and confluent cells. In logarithmically growing KB cells 70% of the cells were viable after 48 hours in 500 μ M fusaric acid. In confluent cell, fusaric acid did not show significant cytotoxic effect, as determined by the fact that 95% of the cells survived 48 hours of treatment with 500 μ M fusaric acid (See Table 4, above). Thus, in contrast to LoVo cells, a significant proportion of the population of growing (70%) cells and virtually all (95%) of confluent KB cells resisted the pronounced cytotoxic action of fusaric acid (See Tables 3 and 4, above).

EXAMPLE 5

Effect of Fusaric Acid on Growth and Viability of Human Breast Adenocarcinoma Cells.

Fusaric acid (500 μ M) rapidly inhibited human breast adenocarcinoma MDA-468 cell growth. After 12-24 hours of treatment with 500 μ M fusaric acid, there was no further increase in cell number. DNA synthesis was completely inhibited (100%) by 24 hr. When treated with 500 μ M fusaric acid, the majority of the MDA-468 cells became granular, showed pronounced cytotoxic effects, many were destroyed and subsequently detached from the culture dish. These floating cells were not viable. Within 30 hours of treatment there was a 65% decrease in cell number. Following removal of the drug after 30 hours of treatment showed that the cell population continued to decline in number. After 96 hours, less than 10% of the original population remained attached to the dish and no change in cell number was noted after one additional week.

As in the case of WI-38, MDA-468 cell viability after fusaric acid treatment was investigated in logarithmically growing and confluent cells. In logarithmically growing MDA-468 cells, less than 20% of the attached cells were viable after 30 hours of treatment with fusaric acid. When the cells were treated for 48 hours, only 0.1% of the cell population survived the pronounced cytotoxic actions of fusaric acid. In confluent cells, fusaric acid showed significant cytotoxic effect as determined by the fact that only 10% of the cells survived 48 hours of treatment with 500 μ M fusaric acid. Thus, MDA-468 cells are extremely sensitive to the cytotoxic actions of fusaric acid in both growing and confluent population of cells in comparison to normal WI-38 lines studied.

Thus, fusaric acid is effective to reduce and control growth of this common type of human malignancy.

EXAMPLE 6

Effects of fusaric acid on growth and viability of other human carcinoma cell types

As in previous examples, the following human cell lines were inhibited by similar concentrations of fusaric acid: Prostatic adenocarcinoma, skin carcinoma, colon carcinoma, liver adenocarcinoma and lung adenocarcinoma. For all these cell types, cell viability decreased by approximately 60% after 48 hours of treatment with fusaric acid.

EXAMPLE 7

Combined effects of fusaric acid and standard chemotherapeutic agents.

Other chemotherapeutic agents such as 5-fluorouracil and/or levamisole in the case of colon adenocarcinoma may be utilized in conjunction with fusaric acid to enhance the effectiveness of therapy. Irreversible cell death and biological alterations induced by fusaric

acid also may be enhanced by using agents from the group consisting of anti-cancer antibodies, radioactive isotopes, and chemotherapeutic agents.

The method of using fusaric acid or picolinic acid topically to treat a variety of viral and spontaneous proliferative diseases in human and animal subjects, as will be described in detail below, can be used in combination with cytotoxic agents selected from the group consisting of chemotherapeutic agents, antibodies, radioactive isotopes, and cytokines (e.g. Interferons), vitamin A, for enhanced activity.

EXAMPLE 8

Treatment of Metastatic Cancer

The subject is a 62 year old Caucasian male with metastatic colon cancer. The subject presented with an enlarged lymph node in the neck. The lymph node was putting pressure on nerves causing a drooping of the subjects right eye lid. Subsequent testing such as CAT scan and MRI indicated that the lymph node was cancerous. Treatment was initiated with 500 mg of picolinic acid in capsule form, by mouth twice daily. Within 72 hours the lymph node was significantly reduced in size upon palpation, with an estimated reduction of over 50% in mass with concomitant lessening of the droop in the eyelid. An aspiration needle biopsy of the lymph node was attempted but had to be repeated due to the fact that the pathologist was withdrawing necrotic tissue from the lymph node. The subject remains on 500 mg picolinic acid twice daily and is tolerating treatment well. The protocol includes increasing the dosage up to 2000 mg per day, or more, if required.

EXAMPLE 9

Fusaric Acid effect on cells with increased P-protein activity.

Multidrug resistance (MDR) is a formidable obstacle to effective cancer chemotherapy. Studies have indicated that MDR is a phenomenon in which resistance to one
5 drug is associated with resistance to a variety of unrelated drugs. Thus, even when a combination of chemotherapeutics is used, patients may exhibit concurrent resistance to some or all of the drugs, leading ultimately to failure of therapy.

One of the primary contributors to MDR is a glycoprotein denoted P-glycoprotein of molecular weight 170 Kdal , also know as P170. P-glycoprotein or P170 acts as a pump,
10 effectively eliminating chemotherapeutic agents from the cell interior to the extracellular space. Although drug-sensitive cells are destroyed during the initial and subsequent courses of chemotherapy, drug resistance cells, containing elevated levels of P-glycoprotein, emerge, multiply and eventually lead to death of the host.

P-glycoprotein, the product of the *mdr-1* gene is a plasma membrane protein. The
15 molecule is composed of 12 transmembrane domains and two binding sites for ATP, which furnishes the energy required for drug elimination. The function of this protein in normal cells is presumably to eliminate naturally occurring toxic compounds. Elevated levels of P-glycoprotein have been associated with multidrug resistance in numerous malignancies, including: colon carcinoma, breast carcinoma, liver, pancreas, lung carcinoma and other
20 tumors.

From the previous information, it is evident that drugs that are not neutralized by the P-glycoprotein mechanism will be of benefit for chemotherapeutic attack of susceptible and MDR-resistant cells. Of considerably interest for this invention is the data showing that

fusaric acid does not induce P170 protein and is effective in controlling growth of cells with high levels of P170 protein. Thus, fusaric acid may have some role in the treatment of tumors which are resistant to MDR-associated drugs.

EXAMPLE 10

5 Use of fusaric acid to reduce the expression of retroviral mRNA levels

By using Kirsten (K) sarcoma retrovirus-transformed NRK cells it was shown in preliminary experiments that fusaric acid reduces the expression of retroviral mRNA levels. Furthermore, it also may be shown that the combination of fusaric acid and interferon-gamma results in a potent inhibition of K sarcoma virus mRNA expression in K-NRK cells.

10 Identification of fusaric acid as a substance that can inhibit expression of mRNA controlled by a retroviral promoter is a great interest because of the importance of retroviruses, such as the human immunodeficiency virus (HIV), in animal and human disease. Although the biology of K-virus and HIV is different, fusaric acid may be effective in controlling HIV viral expression. Furthermore, the combination of fusaric acid plus
15 interferon-gamma may be much more potent in inhibiting HIV expression in human monocytes and other infected cells. Thus, this inventions is not limited to the effects of fusaric acid in K-NRK, cells but is extended to the actions of this agent in other retrovirally infected human and animal cells.

EXAMPLE 11

Treatment of ulcerative lesion with topical picolinic acid

A subject horse had a 3 inch diameter ulcerative lesion on the left side of its neck. The lesion had a papillomatous appearance with bleeding at the tips of the papillae. The lesion was progressive, with total loss of hair over the area. The diagnosis was viral disease, i.e. papilloma virus, complicated by fungal infection. The horse was treated with conventional local antibiotic and chemical therapies for about four months. However, the agents used did not modify the course of the disease.

An aqueous solution of 10% picolinic acid in deionized water was applied every other day with a cotton swab over and around the lesion. The treatment continued for 45 days. The course of the regression of the viral lesion was as follows:

1) after 10 days of treatment, the bleeding papillae suffered central necrosis and the borders of the ulcer acquired the aspect of granulomatous proliferating healing tissue;

2) after 20 days of treatment, the healing lesion began to show hair growth in multiple areas; the diameter of the lesion was reduced to approximately 2 inches and appeared flat and clean of debris;

3) after 30 days of treatment, the lesion was about 1 inch in diameter with abundant hair growth on the borders and on the surface of the lesion;

4) at 45 days the lesion resolved with some scar tissue; hair covered all of the area;

and

5) after three additional months the horse was observed without evidence of recurring disease.

EXAMPLE 12

Treatment of patients with papilloma virus skin lesions

Picolinic acid and its analogues act by chelating metal ions. In the case of the inhibition of viral replication by picolinic acid, the ion involved is zinc, which is essential to maintain the active structure of zinc finger proteins such as E6 and E7 proteins of the human papilloma viruses essential for viral replication.

Five patients ranging in age from 11 years to 52 years and each having at least one common wart induced by human papilloma viruses was treated with a topical preparation of picolinic acid. The topical preparation was either solution of 10% to 20% picolinic acid in deionized water or a topical ointment wherein 10% picolinic acid is incorporated into Aquaphor, i.e. 1 g of picolinic acid in 10 g of Aquaphor. After seven days of application of the solution or ointment, central necrosis of the wart occurred. After approximately 4 to 6 weeks the warts were gone. It should be noted that there was no significant difference observed in the course of disease between the 10% and 20% solutions. However, faster resolution was seen with the ointment and is believed to be due to the continual contact time imparted by the ointment base.

EXAMPLE 13

Treatment of virus-induced plantar ulcer

A 50 year old patient with recurrent plantar wart of about 2 cm in diameter was treated with topical picolinic acid. The patient, a pathologist who had difficulty walking because of the pain caused by the ulcer, had experimented with numerous medications for more than three months without any significant results prior to treatment with the picolinic acid. It is relevant to note that many plantar ulcers are transformed into malignant tumors.

The patient was treated with a solution of 10% picolinic acid in deionized water for one week. Central necrosis was noted. He then was treated with 10% picolinic acid in Aquaphor. The ointment was placed on the ulcer and on a patch. The patch was replaced every 24 hours. After an additional three weeks the plantar ulcer resolved.

5

EXAMPLE 14

Treatment of metastatic disease to the skull from breast cancer

A 73 year old female with metastatic breast cancer to the skin and bone of the skull was treated with a topical preparation of 10% picolinic acid in Aquaphor. The preparation was applied to the cancerous lesions and to a bandage and changed twice daily. The multiple cancer lesions were approximately 1 to 1.5 cm in diameter. The lesions resolved with scar tissue forming after approximately 35 days.

EXAMPLE 15

Treatment of proliferative skin disorders

Several patients suffering from proliferative skin disorders such psoriasis have been included in a recent ongoing study of the anti-proliferative effects of topical picolinic acid. Preliminary information indicates that the picolinic acid has a significant effect in inducing regression of the psoriasis. The patients may be treated with a topical application of approximately 5% to 20% picolinic acid, or a derivative thereof, in an absorption base. Alternatively, the patient may be treated with a solution containing approximately 5% to 20% picolinic acid, or derivative, in deionized water. The topical preparation may be applied twice a day or in an alternative pharmacologically acceptable regimen.

EXAMPLE 16

Treatment of Actinic lesions

Two patients with actinic lesions (average of 5 lesions per patient, each lesion being approximately 3 mm to 5 mm in diameter) were diagnosed as requiring liquid nitrogen removal of the lesions. The patients received a daily application of 10% picolinic acid in Aquaphor. After approximately three weeks of treatment, the lesions were completely cured (eliminated) without any effects on normal skin.

EXAMPLE 17

Treatment of Herpes

The subject was a 58 year old Caucasian male with at least one “cold sore” or common “fever blister”. The lesions was a typical herpes simplex lesion. The subject has a history of such lesions and has treated them with lip balm or Blistex® with only limited symptomatic relief. The subject applied the subject topical antiviral as a 10% aqueous solution. Within twelve hours of the first application, the subject’s lesion began to shrink with a decrease in soreness and pain. After approximately 24 hours from the initial application, the lesion as almost completely healed. He made a third application approximately 36 hours after the first application. Between 36 hours and 48 hours after the initial application, the subject described the fever blister as “gone” and “healed”.

EXAMPLE 18

Treatment of Herpes

The subject was a Caucasian female in her mid-fifties with a long history of recurring, painful herpes simplex lesions described as “fever blisters”. The subject presented with a painful lesion on her lip. She applied a 10% aqueous solution of the subject antiviral to the

lesion approximately three or four times at 12 hour intervals. She reported that the lesion was nearly gone after the third application, but made the fourth application to “make sure”.

EXAMPLE 19

Treatment of Herpes

5 The same female subject of Example 19 reported that should could feel the characteristic “tingling” sensation in her lip that usually preceded the eruption of a “fever blister”. Upon feeling the “tingling”, the subject made one application of a 10% solution of the subject topical antiviral. Within 12 hours, the tingling sensation ceased and there was no eruption of a blister.

EXAMPLE 20

Treatment of Herpes

10 A 47 year old Caucasian female with a history of frequent herpes simplex eruptions she characterized as “cold sores” or “fever blisters”. The subject presented with a rather large, painful blister on her upper lip. The subject applied a 10% aqueous solution of the
15 subject antiviral. Within 12 hours there was a decrease in pain and soreness and she began to experience a drying of the lesion she described as “ a sort of scabbing”. She made a second application approximately 12 hours later. The lesions continued to resolve. At approximately 36 to 48 hour after the initial application the lesion was described as “pretty well gone”.

EXAMPLE 21

Treatment of Herpes

20 The subject was a 17 year old Caucasian male who presented with numerous painful white sores in his mouth and throat areas typically described as “stomach sores” or herpes.

10 The subject suffered from the lesions for approximately two days. He could barely eat solid
food due to the discomfort and pain. Before bedtime on day two, the subject took
approximately 1/2 ounce of a 10% aqueous solution of the subject antiviral and swished it
around in his mouth and spit it out. Upon awakening, approximately 8 hours later, the subject
5 reported his mouth did not hurt, but that the sores were still there. He applied a second dose
in a similar manner that morning. That evening he reported that he could eat without pain, but
felt that one or two "spots" were still tender. He made no more applications. At
approximately 24 hours from the first application, the subject reported that his mouth was
healed.

10 EXAMPLE 22

Treatment of Upper Respiratory Infection

A 21 year old male college student presented with nasal congestion, slight fever,
cough and sore throat. The subject began treatment with nasal spray containing 3mM
picolinic acid in physiologic saline vehicle. The subject used one or two sprays in each nostril
15 after evening meal and before bedtime. Upon arising, the subject had no fever or sore throat
symptoms and the cough and post-nasal drainage had resolved.

EXAMPLE 23

Use Of Picolinic Acid For Orocutaneous Herpes Simplex

20 Data on over 60 patients who have used a picolinic acid ointment applied to
orocutaneous herpetic lesions. The study was focused on the first 48 hours following
treatment of a herpes outbreak with 10% picolinic acid in an ointment (PA Ointment). All
subjects in the test group who experienced the initiation of a herpes eruption were treated
within 24 hour with PA Ointment. Data collected over the first 8 days were: level of pain and

discomfort during the first 24 hours; inflammation at the eruption site within 24 hours; if advanced infection and blisters were present, collapse of blisters within 2 hours; perception of pain; and attenuation of infection within 48 hours were recorded.

All of the patients universally experienced resolution of pain, swelling, inflammation, and vesicle formation within 24 to 48 hours of initiating its use. In previous episodes, all of these patients had failed adequate symptomatic control of their viral eruptions with conventional therapy. There was no toxicity to the normal or viral infected skin areas noted in this study.

EXAMPLE 24

Treatment of Chickenpox

The subject was a 10 year old female Caucasian presenting with typical chickenpox. The subject developed the typical rash on her torso, particularly her back and abdomen. When the rash was still in its early stages, before full-blown blistering occurred, groups of the lesions were marked by encircling with an indelible marker. A 10% solution of the subject antiviral was applied to the lesions inside the marked areas. These lesions did not erupt into blisters and the subject reported that the treated areas did not itch like the others.

ILLUSTRATIVE PREPARATIONS CONTAINING METAL CHELATING PICOLINIC ACID AND DERIVATIVES FOR THE TREATMENT AND PREVENTION OF SPECIFIC

DISEASE STATES

EXAMPLE 1

Topical or intravaginal preparation of picolinic acid in an absorption base

A topical or intravaginal preparation of picolinic acid in an absorption base is made by incorporating 0.001% to 99.9%, preferably 1% to 50%, most preferably 5% to 20% picolinic

acid into an absorption base. An absorption base generally is an anhydrous base which has the property of absorbing several times its weight of water to form an emulsion and still retain an ointment-like consistency. Absorption bases may vary in their composition but generally are a mixture of animal sterols with petrolatum, such as Hydrophilic Petrolatum, U.S.P. The most common commercially available products are Eucerin and Aquaphor (Beiersdorf) and Polysorb (Fougera). One preferred embodiment of the topical preparation is made by dissolving 10% picolinic acid in deionized water and then incorporating the solution into an equal amount of Aquaphor, on a wt/wt basis. Further, the picolinic acid or derivatives can be incorporated into a balm or stick for application to the lips to treat herpes infections. It will be appreciated that picolinic acid derivatives can be used in place of the picolinic acid in the topical preparation. It will be appreciated that an appropriate concentration of a substituted picolinic acid derivative can be used in place of the picolinic acid without departing from the scope of the invention. It will be appreciated that such preparations can be used to treat topical conditions such as virus infections, fungal infections, susceptible bacterial infections, radiation assault, including ultraviolet, medical or atomic radiation, skin cancers or any other condition mediated by the above described mechanisms.

EXAMPLE 2

Picolinic acid solution

Picolinic acid can be employed topically, for vaginal installation, for inhalation or as a mouthwash as a 0.001% to 99.9%, preferably 1% to 50%, most preferably 5% to 20% aqueous solution. One preferred embodiment of the solution is prepared by dissolving an appropriate amount of picolinic acid in an appropriate amount of deionized water to form a 10% solution. The preparation can be used in any pharmaceutically acceptable manner

including topically, orally, on the mucosa and so forth. It will be noted that picolinic acid derivatives can be used in place of the picolinic acid, if desired. For inhalation purposes, the solution may be atomized with the use of an appropriate device.

As stated above, it is likely that picolinic acid will interfere with the replication of the retroviruses by chelating zinc and preventing the activity of certain zinc finger proteins. Therefore, a suitable preparation of a chelating material, for example, picolinic acid or derivative may be used for vaginal application to prevent infection with any virus containing zinc finger proteins as an essential component of the viral replicating machinery, i.e. transcription factors. Such viruses include, but are not limited to, human papilloma viruses (E6 and E7 zinc finger proteins) and the AIDS virus (tat protein). As explained above, the picolinic acid and substituted derivatives thereof are used to attack the p7 protein having two zinc finger segments which is found in the HIV virus which causes AIDS, which is essential for packaging RNA in the viral particles.

The preparation may be prepared by incorporating approximately 5% to 20% picolinic acid in a suitable base, such as Aquaphor, and instilling the ointment vaginally before coitus. It also may be possible to prepare a douche of approximately 0.001% to 99.9%, preferably 1% to 50%, most preferably 5% to 20% picolinic acid in deionized water and used before and after coitus. Such preparations may be used prophylactically to prevent infection with these viruses.

Furthermore, the preparations may be used vaginally to treat the uterine cervix infected with papilloma virus.

A condom containing approximately 5% to 20% picolinic acid or derivative may be used to prevent replication of the viruses in the vaginal and cervical cells in the event the

condom fails or ruptures. It will be appreciated that an appropriate concentration of a substituted picolinic acid derivative can be used in place of the picolinic acid without departing from the scope of the invention.

EXAMPLE 3

Ocular Preparation

A preparation of picolinic acid or a derivative thereof can be prepared for the treatment of ocular herpes or other retroviral infections of the eyes. The topical or intraocular ophthalmological preparation includes approximately 0.01% to approximately 5% picolinic acid or one of its substituted derivatives in an appropriate, ion-free vehicle, such as methylcellulose. The preferred embodiment would include 0.01% of picolinic acid or derivative for topical ophthalmological application. However, the invention is intended to include a broader range of concentrations of picolinic acid or derivative thereof. It will be appreciated that an appropriate concentration of a substituted picolinic acid derivative can be used in place of the picolinic acid without departing from the scope of the invention.

EXAMPLE 4

Acne Formulation and Sunburn Treatment

A preparation useful in the treatment and control of acne comprises approximately 7.5% to 10% picolinic acid, by weight, in a suitable topical lotion. The acne preparation can include approximately 1% to approximately 99% picolinic acid, derivative or analog thereof. A preferred range is approximately 5% to approximately 15%. The lotion is applied to the skin two or three times daily.

The above described lotion also can be used to control the symptoms of sunburn.

EXAMPLE 5

Inhalation or Intranasal Formulation

A product suitable for intranasal administration for treatment of upper respiratory diseases includes approximately 3mM picolinic acid in a suitable isotonic vehicle. One example is 3 mM picolinic acid in Ocean® Nasal Mist (Fleming & Co., St. Louis, Mo.). The intranasal solution in a range between 0.01 mM to 50 mM, preferably 0.1 mM up to 20 mM picolinic acid or greater.

Likewise, a solution for pulmonary inhalation is prepared by adding picolinic acid to normal saline for nebulization, the resulting solution being in a range of 0.001% to 50% picolinic acid, derivative or analog in saline or sterile distilled water for nebulization.

EXAMPLE 6

SYSTEMIC ADMINISTRATION

A systemic preparation of a picolinic acid, its derivatives or analogs containing approximately 1% to 100% active ingredient may be administered orally, intravenously or by any acceptable route for the treatment of cancer and systemic infections. For example, picolinic acid prepared in 00 gelatin capsules at 500 mg per capsule has been shown to be effective in the control of metastatic cancer. The preparation can be provided as a flavored oral solution. Likewise, an injectable form may be prepared.

As set out above, the safe and effective daily systemic dose may range for 250 mg to 140 grams for a 70 Kg subject, with the preferred range being 250 mg to 6 grams, and the most preferred dose being 500 mg to 2000 mg.

[illegible][illegible]

	0.7	0.8	0.9	1.0	1.1	1.2	1.3	1.4	1.5	1.6	1.7	1.8	1.9	2.0	2.1	2.2	2.3	2.4	2.5	2.6	2.7	2.8	2.9	3.0	3.1	3.2	3.3	3.4	3.5	3.6	3.7	3.8	3.9	4.0	4.1	4.2	4.3	4.4	4.5	4.6	4.7	4.8	4.9	5.0	5.1	5.2	5.3	5.4	5.5	5.6	5.7	5.8	5.9	6.0	6.1	6.2	6.3	6.4	6.5	6.6	6.7	6.8	6.9	7.0	7.1	7.2	7.3	7.4	7.5	7.6	7.7	7.8	7.9	8.0	8.1	8.2	8.3	8.4	8.5	8.6	8.7	8.8	8.9	9.0	9.1	9.2	9.3	9.4	9.5	9.6	9.7	9.8	9.9	10.0
0.7	0.8	0.9	1.0	1.1	1.2	1.3	1.4	1.5	1.6	1.7	1.8	1.9	2.0	2.1	2.2	2.3	2.4	2.5	2.6	2.7	2.8	2.9	3.0	3.1	3.2	3.3	3.4	3.5	3.6	3.7	3.8	3.9	4.0	4.1	4.2	4.3	4.4	4.5	4.6	4.7	4.8	4.9	5.0	5.1	5.2	5.3	5.4	5.5	5.6	5.7	5.8	5.9	6.0	6.1	6.2	6.3	6.4	6.5	6.6	6.7	6.8	6.9	7.0	7.1	7.2	7.3	7.4	7.5	7.6	7.7	7.8	7.9	8.0	8.1	8.2	8.3	8.4	8.5	8.6	8.7	8.8	8.9	9.0	9.1	9.2	9.3	9.4	9.5	9.6	9.7	9.8	9.9	10.0	